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Target alternative vaccine safety testing strategies for pertussis toxin

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Abstract

All acellular pertussis (aP) vaccines in use contain chemically inactivated pertussis toxin (PT). The finding that mice, naturally resistant to the effects of histamine, become sensitive upon injection of minute amounts of PT, led to the development of the test for residual PT known as the histamine sensitization assay (HSA). The HSA used by U.S.-licensed manufacturers is a limit test that shows that the residual bioactivity of PT in a single human dose of vaccine is below a threshold. Limit tests do not allow quantitative measurement. When the method is newly established at the point of use, three or more dilutions of pure PT are used to verify that mice injected with the vaccine came from a shipment that have sensitivity consistent with historical values. Sensitizability is expressed as an HSD_{50} (the dose that sensitizes 50% of a group of mice). However, once linearity of the dose response has been demonstrated, the assay may be simplified so as to include in each test only a single control group injected with PT. This assay simplification constitutes an example of the so-called "consistency approach." A Japanese variant of the HSA uses a drop in body temperature as a nonlethal alternative index of PT-mediated sensitization and can provide a quantitative estimate of the residual PT activity of a vaccine. However, the advantage of a quantitative method is not obvious, because the amount of PT that is unsafe for humans is unknown. In addition, although the use of a nonlethal endpoint constitutes an important refinement, the need for a reference group in the test to obtain a quantitative estimate increases the number of animals required, relative to the number used in a simplified limit test. Moreover, the nonlethal endpoint might be adapted to the limit test format, and important steps have been taken in this regard. Finally, one option under early evaluation is the possibility of using the results from two *in vitro* assays, an enzymatic activity assay and a binding assay, to replace the HSA.

Keywords: pertussis; vaccine; testing

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1. Background

Pertussis (whooping cough) continues to be a worldwide public health concern even in countries with high vaccination coverage [1]. Public unease about the side effects of whole-cell pertussis (wP) vaccines prompted the development of acellular pertussis (aP) vaccines in the late 1970s and early 1980s. These vaccines contain up to five purified pertussis antigens and are less reactogenic than wP vaccines [2]. One of these antigens is pertussis toxin (PT), which in chemically detoxified form is included in all U.S.-licensed vaccines that contain an aP component. Thus, a test that measures the residual PT toxicity of chemically inactivated PT and the possible reversion of toxoid to toxin upon storage is important to ensure vaccine safety.

PT is a typical AB toxin (one **A**ctive protomer plus a pentameric **B**inding oligomer). The B oligomer binds to various (but mostly unidentified) glycoconjugate molecules on the surface of a number of mammalian target cells. The enzymatic activity of PT resides in the A protomer. Once in the cell cytosol, A hydrolyzes cellular NAD and transfers the released ADP-ribose to a specific cysteine residue near the C-terminus of the α subunit of heterotrimeric G-proteins of the Gi family in mammalian cells. This modification alters a number of Gi protein-coupled signaling pathways in a variety of cell types [3].

1.1. Toxicity of PT for humans

Ideally, no toxic material should remain in vaccines; however, when the antigen itself is a toxin, the manufacturer is presented with the unique problem of achieving acceptably low levels of biological activity by detoxification while preserving a molecule with satisfactory levels of protective immunogenicity. Clinical studies in humans to quantitatively measure toxicity are obviously impossible to perform on ethical grounds. Additionally, detection of residual toxicity will be influenced by the assay method.

Whole-cell pertussis vaccines, considered generally safe and effective [1], have been in use for many years (until 1998 in the United States) and reportedly may contain up to 300 ng of active PT per dose [4]. Moreover, in a limited study of some therapeutic properties of PT, the intravenous injection of substantial quantities (up to 1 μg per kg of body weight) of active toxin into adult human volunteers failed to cause conspicuous adverse reactions [5].

Rather than basing it on known toxicity in humans, the acceptance criterion for residual PT in aP vaccines licensed in the United States was adopted on the basis of a set of principles that have become known as the "consistency approach" [6]. In this approach, product characteristics somewhat predictive of vaccine safety and efficacy are measured by analytical methods on vaccine batches after licensure. Similarity between these characteristics and those of batches demonstrated to be safe and effective in clinical trials is sought. This design limits the scope of a test result to its compatibility with a product of acceptable quality in the clinic.

2. Use of the histamine sensitization activity of pertussis toxin in a toxicity test

It was reported 62 years ago [7] that the treatment of mice with wP vaccine increased their sensitivity to the lethal effect of histamine by approximately 100-fold. Advances in the immunochemical characterization of *Bordetella pertussis* allowed investigators in the field to subsequently attribute this effect to PT.

The numerous names that PT was given in the past (among others, leukocytosis-promoting factor, histamine-sensitizing factor, and islet-activating protein) reflect the diverse cell types for which it may be noxious [8]. In principle, each of these and other effects, such as the growth in clumps of Chinese hamster ovary (CHO) cells cultured in the presence of the toxin, could be the basis for a test for residual activity; however, only one assay could detect residual PT in a matrix containing aluminum adjuvants and preservatives at an adequate level of sensitivity for aP vaccines: the histamine sensitization assay (HSA) [9]. Various other assays, such as the leukocytosis-promoting assay (LPA) and the CHO-cell test [10], have been explored as alternatives to the HSA to detect residual active PT (**Table 1**). The LPA has shown poor reproducibility and large interlaboratory variation. Moreover, test sensitivity diminishes if the test sample must be injected using the intraperitoneal route instead of the intravenous route, such as when the presence of adjuvant precludes the use of intravenous injection. The CHO-cell test is useful for the determination of residual PT activity in pre-adsorbed bulk components and when the test vaccine in final bulk can be assayed at a higher dilution (as is the case with wP). However, the test is not suitable for testing for the

presence of residual active PT in final bulks of aP vaccine, because the aluminum salt used as an adjuvant or the presence of a preservative may cause CHO cell death at lower vaccine dilutions [9]. In addition, the correlation of this test with *in vivo* toxicity has been questioned [11].

Table 1. Some characteristics of three assays for active PT

	CHO CELL ASSAY	LEUKOCYTOSIS PROMOTION	HISTAMINE SENSITIZATION
DIFFICULTY	+ (*in vitro*)	+++ (*in vivo*)	+ (*in vivo*)
SENSITIVITY	≅ 135 pg/mL	8-40 ng (i.v.) ≅ 200 ng (i.p.)	≅ 31 ng (HSD_{50})
COST	$	$$	$$$
SUITABILITY FOR FINAL BULK	PT–unrelated toxicity of some vaccines for the cells	Some adjuvants are toxic for the mouse via i.v.; i.p. route less sensitive	YES

The HSA is based on the principle that mice, which are usually resistant to the lethal effect of histamine, become sensitive upon injection of PT. The HSA is able to detect minute amounts of active residual PT, and it has therefore been chosen as the toxicity release test for aP vaccine. In this assay, groups of mice are injected concurrently with variable doses of pure active PT and the vaccine being tested. A few days later, animals are challenged with histamine [9]. Mice that received enough PT to be sensitized will go into hypovolemic shock. This is a condition where the heart is unable to supply enough blood to the body because of circulatory failure or inadequate blood volume [12]. Although most of the sensitized mice die of circulatory collapse within one hour, deaths due to histamine are recorded after 24 hours. The purpose of the dilution series of PT is to verify that the animals injected with the vaccine in a given day show sensitizability consistent with historical limits. Such sensitizability is defined in terms of an HSD_{50} (median histamine-sensitizing dose: the dose that sensitizes 50% of a group of mice to the lethal effects of histamine). The HSA can be used to validate detoxification procedures for PT, in terms of both residual toxicity and reversion, and to check other pertussis antigens for contamination with active PT.

2.1. Toxicity testing of whole-cell pertussis vaccines

The 2007 WHO recommendations for wP vaccine [13] describe a variation of the classical mouse weight gain test to assess vaccine toxicity, which was limited to record the weight and general health status of mice [14]. Blood is drawn on Day 7 post-injection from the mice injected with the vaccine being tested. An increase in the leukocyte count is considered to reflect the presence of PT in the vaccine. Additionally, the recommendations suggest the possibility of measuring PT in vaccines using the CHO cell assay [10] or the HSA test.

Some laboratories have used the HSA to measure residual active PT in wP-containing vaccines relative to a reference vaccine to which a histamine-sensitizing unitage (HSU) has been assigned [15]. However, the European Pharmacopoeia [16] monograph for wP (0161, Pertussis Vaccine, Whole Cell Adsorbed) indicates that a semiquantitative CHO-cell assay should be used to assess the presence of active PT. Moreover, although the WHO Recommendations state that the levels of PT should be monitored during the detoxification processes, as well as when the methods used for detoxification are being validated and consistency of manufacturing is being established, they do not suggest limits [13].

3. Factors that affect the enhancement of histamine-mediated shock by PT in the mouse

In the mouse, the treatment with PT alters or inhibits a basic physiological function that normally acts to protect against the toxic effects of many substances that kill the animal through hypovolemic or low-resistance shock. One of these substances is histamine.

Many factors influence the histamine-sensitizing activity of PT, including the strain, age, and gender of the mice used in the assay; the route of administration of the preparations; the amount of histamine used for the challenge; and a number of environmental factors that have not been well characterized.

3.1. Mouse strain

Several mouse strains with a Swiss-Webster ancestry are highly susceptible to histamine sensitization by PT. Their sensitivity tends to increase 50- to 100-fold upon PT injection. All Swiss mice come from two male and seven female albino mice imported into the United States by Dr. Clara Lynch of the Rockefeller Institute in 1926 [17]. These mice came from a noninbred stock in the laboratory of Dr. de Coulon, Centre Anticancereux Romand, Lausanne, Switzerland. On the other hand, a number of strains, both inbred and outbred, are poorly sensitized.

3.1.1. Responsive strains

Among the commercially available strains most responsive to sensitization by PT are those commonly referred to as ICR, CFW, and NIH, in current use in the United States.

Hilltop's Hla®: (ICR)CVF® Swiss mice originated at the Institute for Cancer Research (ICR) in Philadelphia. The Hauschka/ICR strain was initiated in 1948 from *Swiss* mice of Rockefeller origin. From the Institute of Cancer Research, the mice then went to Roswell Park Memorial Institute and then to Charles River Laboratories, Inc., in 1959. In the 1970s, the mice were obtained by I.C.I. Americas, Inc., and rederived at Hilltop Lab Animals, Inc. In 2002, the mice were rederived by cesarean section [18].

Crl:CFW®(SW)BR resulted from the selective inbreeding by Dr. Leslie Webster using foundation animals from a large colony of Swiss mice maintained at the Rockefeller Institute. The strain went to Carworth from The Rockefeller Institute (now University). It was highly inbred when acquired by Carworth. Current mice come from a selected single pair derived in 1974 by cesarean section and progeny outbred from that point [19].

CR:NIH(S) mice originate from N:NIH(S) (known in the old literature as NIH-BS, NIH-BXS, NIH[SW], and NIH). This strain was derived in 1936 from 12 N:GP(S) mice with Swiss-Webster ancestry. The mice were maintained by multiple-branched inbreeding by brother-sister mating until a change to restricted random breeding in the mid-1960s. In 1973, N:NIH(S) mice were specific-pathogen-free derived and started being bred in a tightly controlled closed colony [20]. In 1986, mice started being bred by the Frederick National Cancer Research Facility at Fort Detrick, Maryland.

Breeding procedures may seriously alter mouse sensitizability (**Table 2**).

3.1.2. Nonresponsive strains

Although outbred and inbred strains differ in susceptibility to histamine challenge after PT sensitization, only C3H/HeJ and CBA/J mice among 14 inbred strains were completely resistant to histamine sensitization at all doses tested using a histamine challenge dose as high as 100 mg/kg [21].

3.1.3. Genetics of histamine sensitization

When administered *in* vivo, PT enhances vascular permeability, which is manifested by a concomitant increase in sensitivity to a variety of agents and treatments affecting the vascular bed. The marked differences between inbred mouse strains to the induction of histamine sensitization by pertussis vaccine implied that there was a genetic basis for this response [20]. Inbred strains of mice differ in susceptibility to vasoactive amine challenge after PT sensitization in that genetically susceptible strains die from hypotensive and hypovolemic shock, whereas resistant strains do not [12]. All the observations seemed to suggest that sensitization to histamine by PT in mice was regulated by a single dominant autosomal gene [22]. This was later confirmed [21]. Histamine sensitization by PT, as measured by hypersensitivity following vasoactive amine challenge, is genetically controlled by the *B. pertussis*-induced histamine sensitization locus (Bphs). Bphs was mapped to the central region of mouse chromosome 6 [23], adjacent to genes belonging to the tumor necrosis factor (TNF) receptor superfamily [24]. Bphs has been identified as the histamine H_1 receptor (Hrh1). It has been reported that the contribution of Bphs/Hrh1 to the overall genetic control of responsiveness to PT is limited to susceptibility to histamine hypersensitivity and enhancement of antigen-specific delayed-type hypersensitivity responses [25].

Table 2. Median histamine sensitizing doses (HSD_{50}) of PT, ICR, and NIH mice of two sources

STRAIN	SUPPLIER	HSD_{50}
ICR	Taconic	280 ng[a]
ICR	Hilltop	16 ng[b]
NIH	FCRF	23 ng[c]
NIH	HARLAN	225 ng[d]

[a]Geometric Mean (GM) of 2 assays
[b]GM of 5 assays
[c]GM of 13 assays
[d]GM of 12 assays

3.2. Other factors

Mouse age has also been suggested to affect the histamine-sensitizing effect of PT, although this factor seems to be less critical for some strains. Nevertheless, for example, the sensitivity of the CFW strain is thought to be more uniform among 5- to 7-week old mice than among younger animals [12]. It has been also reported that female mice are more sensitive to histamine sensitization than males, although the detection of this difference requires large numbers of animals [26,12]. The amount of histamine used for the challenge also affects the outcome of the assay. It has been reported that the number of deaths due to sensitization does not increase monotonically with the histamine challenge dose [27]. Therefore, the use of a fixed sensitizing dose of PT-containing material and variable histamine challenge doses was abandoned.

To control for the effect of environmental factors not well understood, the use of a true randomization procedure to allocate the mice to treatment groups and for positioning cages on shelves is strongly recommended.

4. HSA test designs for the testing of aP vaccines

4.1. United States

The standard HSA design used for aP vaccines licensed in the United States is as follows. Groups of 20 (4–5 wks, 15–20 g, female) mice are injected intraperitoneally with 0.5 mL of the undiluted vaccine being tested, phosphate-buffered saline (PBS) containing 0.2% gelatin (diluent/negative control), or dilutions of PT (positive control). The PT dilutions are selected to cover a sensitizing range that will predictably include the HSD_{50}. Five days after injection, all mice are challenged intraperitoneally with 1 mg of histamine base, as the monohydrated diphosphate, diluted in 0.5 mL of PBS. Within 1 hour, most of the sensitized mice will die of circulatory collapse and will be removed. Deaths are recorded at 24 hours. The assay is considered valid if there are no indications of departure from linearity of the log dose-probit line ($p<0.01$) and if the HSD_{50} is bracketed by the dilutions used and falls between 10 and 100 ng. In addition, no more than 10% of mice should die in the negative/diluent group.

A vaccine is considered acceptable if one undiluted single human dose of 0.5 mL sensitizes no more than 10% of mice injected. If the vaccine fails to meet the criterion in a first test, to be recommended for release it should pass in two additional, consecutive and independent, tests.

Results presented in **Tables 2–6** were obtained in experiments performed using this basic design.

4.1.1. Derivation of limits of mouse sensitizability by PT for assay validity

The HSD_{50} of pure PT was estimated in multiple tests for three strains of mice reputed to be of suitable sensitizability to histamine (see 3.1.1 above). At least 10 valid assays per strain were used to estimate sensitizability limits. Linear models including all 36 results and dose, strain, and testing date as variables detected a significant difference in HSD_{50} among the three strains (**Table 3**). This result was confirmed by analysis of variance ($p < 0.0025$). CFW mice had a higher HSD_{50} than both ICR and NIH strains (**Table 3**), and the HSD_{50}s of the ICR and NIH strains did not differ significantly from each other.

Table 3. Median histamine sensitizing doses (HSD_{50} in ng) for three strains of mice

STRAIN	n	GM[a] HSD_{50}	MEAN LOG	S.D. LOG
CFW	13	45.0	1.6523	0.1789
ICR	11	26.7	1.4260	0.1436
NIH	12	22.8	1.3582	0.2649

[a]Geometric mean

Because the three strains have been considered sensitizable, the upper validity limit was established using the HSD_{50} of the strain with the lowest sensitizability (CFW) and the lower validity limit was established using the HSD_{50} of the strain with the highest sensitizability (NIH):

- HSD_{50} upper validity limit (antilog 97.5 percentile of HSD_{50} distribution for CFW strain): $10^{[1.6523 + 1.96(0.1789)]}$ = 100.7 ng, rounded to 100 ng
- HSD_{50} lower validity limit (antilog 2.5 percentile of HSD_{50} distribution for NIH strain): $10^{[1.3582 - 1.96(0.2649)]}$ = 6.9 ng, rounded to 10 ng

Alternative assay designs have been allowed if they are at least demonstrably equivalent in their capacity to detect residual active PT in aP vaccines. The setting of a lower validity limit by a manufacturer is optional, because the regulatory interest is to identify a shipment of low sensitizability, that is, one showing a higher-than-usual HSD_{50}.

Tables 4 and 5. Histamine sensitization of mice by aP from two manufacturers (A&B) spiked with 22 ng PT per 0.5 mL

Table 4

PT Conc.[a]	Deaths @ 24h Injected mice
125 ng	19/20
25 ng	11/20
5 ng	0/20
0 ng	0/20

[a]Assay HSD_{50} = 25.9 ng

Table 5

Vaccine[a]	Deaths @ 24h Injected mice
A	0/10
A + PT	8/10
B	0/10
B + PT	6/10

[a]0.5 mL per mouse

4.1.2. Assay sensitivity

The current HSA used by U.S.-licensed aP vaccine manufacturers is a limit test designed to demonstrate that the PT residual bioactivity in a single human dose of vaccine is below a defined threshold. Limit tests do not allow a quantitative measurement of the amount of residual active PT in the vaccine. Notwithstanding the successful use of this approach since at least 1991 in the United States, the international biologics community has expressed concern about the nonquantitative nature of the test. Moreover, despite the uncertainty of the amount of residual PT that detoxification procedures should achieve, a recent WHO informal consultation (November 9-13, 2009) on acellular pertussis vaccine recommended the use of the International Standard PT to calibrate the sensitivity of the assay system in IU of PT bioactivity (unpublished). For the sake of international harmonization, we at CBER have explored the possibility of estimating the amount of residual active PT that would cause the test outcome that has been considered as compatible with aP acceptance for almost 20 years.

For example, the HSD_{10} (two mice sensitized from a group of 20) of PT in a shipment of mice with an HSD_{50} of exactly 100 ng (the minimal acceptable value: 10 mice sensitized from a group of 20) has a calculated value of 51.3 ng (by probit analysis). For this modeling, three doses, equally spaced with a factor of five, were used. The

proportions of response to 500 ng and 20 ng were imputed to be 0.001 and 0.999, respectively (to prevent undefined probit values at the asymptotes while ensuring curve symmetry).

Table 6. Proportion of mice of three strains sensitized to histamine by 100 ng PT

Assay	NIH		CFW		ICR	
	HSD_{50} in ng	# Dead at 100 ng	HSD_{50} in ng	# Dead at 100 ng	HSD_{50} in ng	# Dead at 100 ng
1	23.5	18/20	6.29	15/20	23.2	16/20
2	13.0	20/20	6.86	18/20	37.6	14/20
3	17.4	19/20	5.26	20/20	16.2	19/20
4	51.8	18/20	13.4	20/20	11.5	14/20
5	16.2	20/20	10.1	15/20	7.13	17/20
6	26.0	19/20				
7	21.8	20/20				
8	16.2	17/20				
9	7.3	20/20				
10	15.3	20/20				

On the other hand, to measure experimentally the quantity of active PT that must be present in a vaccine for it to fail the acceptance criterion, 3 mL of four vaccine lots from two different manufacturers were pooled separately to give a total of 12 mL of vaccine per manufacturer. Six milliliters were spiked with 2 μL of PT lot 1 @ 132 μg/mL to achieve a concentration of 22 ng of PT per 0.5 mL of vaccine. Twenty NIH mice were injected with the preparations, and the HSD_{50} of the PT used was estimated as per 4.1. The outcome of this experiment is shown in **Tables 4 and 5**. Results indicate that 22 ng of residual PT or even less in a vaccine matrix will make the vaccine fail the HSA.

4.2. WHO guidelines

The assay design description in the WHO guidelines for aP vaccines [9] indicates that groups of 10 to 20 mice of a set strain, gender, and age are allocated at random to three or more serial dilutions of pure PT and to one or more dilutions of the vaccine final bulk being tested. Another group, the negative control, is injected with diluent. After housing the mice for 4 or 5 days at random positions in the animal room, groups are challenged with histamine in the same order in which they were placed on the shelves. Deaths are recorded 24 hours after challenge.

For the assay to be valid, mice injected with diluent should not show significant sensitization to histamine, and the HSD_{50} of PT for the strain used in the assay should be within an acceptable range. Experience has shown that a small percentage of mice (i.e., less than 5%) in the diluents control group may die following histamine challenge. Thus many laboratories will consider a test valid if there is no more than one death in this group. This clarification to the published guidelines was recommended at a recent WHO informal consultation on acellular pertussis vaccine (November 9-13, 2009, unpublished). On the other hand, a footnote in the current guidelines indicates that a strain is adequate for the test if the point estimate of HSD_{50} in each test is below 50 ng; however, it also indicates that variability of the estimate has to be taken into account when setting validity limits. The procedure described in the European Pharmacopoeia monograph (see Section 4.3) uses the point estimate only to define suitability when the assay is performed periodically for antigen concentrates. The HSD_{50} should be calculated using a suitable procedure, such as probit analysis, and the log dose-probit curve should not show significant departure from linearity ($p < 0.01$). The assay can be simplified by including a single positive control once linearity has been demonstrated over time.

Acceptable residual activity is commonly expressed as the maximal proportion of mice that are sensitized to histamine by a dose (usually a single human dose) of the bulk. Activity should not exceed that of lots shown to be

safe in clinical studies. If the vaccine fails to meet the acceptance limit in a first stage, it should meet the criterion in two additional, consecutive and independent, assays to be deemed acceptable for marketing.

If necessary, the amount of residual PT in vaccines being tested can be expressed as an HSD_{50}. However, most currently licensed aP vaccines likely lack enough PT residual activity to allow meaningful calculation of an HSD_{50}. The option of expressing residual activity in terms of an HSD_{50} may therefore be eliminated in future WHO documents.

4.3. European pharmacopoeia

The most recent edition of the European Pharmacopoeia (2010) includes monographs for Pertussis Vaccine (Acellular, Component, Adsorbed; Monograph 1356) and Pertussis Vaccine (Acellular, Co-purified, Adsorbed; Monograph 1595). The Purified Antigenic Fraction (Monograph 1595) or Purified Component (Monograph 1356) should be tested for residual pertussis toxin or its absence, respectively [16]. The test for residual pertussis toxin consists of the intraperitoneal injection of the fraction on test in a volume of no more than 0.5 mL. Three groups of 5 histamine-sensitive mice (18-26 g) each are administered the fraction diluted in PBS containing 0.2% gelatin, in a way to achieve a graded response. Diluent is injected into a fourth group. Five days afterward, mice are challenged intraperitoneally with 1 mg of histamine base in a volume of no more than 0.5 mL. Deaths are recorded in 24 hours. The result is expressed as the weight or volume of material that sensitizes 50% of the mice and is calculated by an algorithm such as probit analysis. The activity of the fraction should not exceed that of fractions used to prepare lots safe in the clinic (for Monograph 1595). The same test applies to the final bulk or final lot of vaccine.

The test on antigenic fraction states that the sensitivity of the strain of mice should be verified at suitable intervals by showing that more than 50% of mice are sensitized with 50 ng of PT. None of the negative controls injected with diluent should die. Absence of reversion to toxicity is tested by means of the same assay design, but using vaccine (final bulk or final lot) that has been incubated for one month at 37ºC.

For adsorbed pertussis vaccine (Acellular, Component, Monograph 1356), the test includes a single test vaccine group of mice. Each group will receive either (1) the equivalent of one human dose of the purified component administered intravenously or (2) the equivalent of twice the human dose administered intraperitoneally, in a volume of PBS–gelatin not to exceed 0.5 mL. If no animal dies in the test upon histamine challenge, the preparation complies with the specification. For final bulk or final lot of vaccine, a volume equivalent to twice the single human dose is injected intraperitoneally in at least five mice, and an equivalent dose of vaccine incubated for one month at 37ºC is administered to a second group. All groups, including a negative control, are challenged intraperitoneally five days after injection with 2 mg of histamine base in a volume not exceeding 0.5 mL. No control mouse should die for the assay to be valid, and no test mouse should die for the assay to pass. The sensitivity of the strain of mice should be verified at suitable intervals by showing that more than 50% of mice are sensitized with 50 ng of PT. If one mouse dies in either test group, the test could be repeated. The assay passes if the total number of mice that die in all assays does not exceed 5%. All other test characteristics are similar to those already described.

5. Changes to the HAS that impact the use of animals

5.1. Inclusion of a PT standard in the lethal endpoint design

In the U.S. test design, one or more dilutions of a pure PT preparation are included in every assay to verify the sensitizability of the shipment of mice used in each HSA test. The current material, U.S. PT control preparation, Lot 1, consists of purified toxin at 132 μg/mL in phosphate buffer with 250 mM NaCl, pH 7.6, containing 50% glycerol. The preparation is stored at –20ºC conditions, under which it has remained stable after more than 13 years of use.

The design of the basic U.S. assay as a nonquantitative test has been questioned on the grounds of classical bioassay principles that recommend the expression of bioactivity of a test sample in terms relative to that of a reference material to reduce assay variability [28]. Nevertheless, the International Conference on Harmonization [29] allows the use of either quantitative or limit tests for the control of impurities, such as residual active PT.

In a collaborative study that was used as the basis for the adoption of the First International Standard of PT [30], the HSD_{50} estimate of the proposed standard was shown to vary between 39 and 1287 ng among the participants returning evaluable data. The study concluded that expressing PT activity in relative terms improves agreement

between laboratories about tenfold. However, a similar result might have been obtained by standardizing critical variables to ensure comparable assay sensitivity. For example, the design of the study allowed each laboratory to use the strain of mouse customarily employed. When the same mouse strain, or strains of similar sensitizability are used by two laboratories, variability may be substantially lower. For example, results submitted to the Center for Biologics Evaluation and Research (CBER) by a U.S.-licensed manufacturer in an 8-year period indicate that the HSD_{50} of the same PT control for the same mouse strain used during the entire period was 12.7 ng (10.9-14.7; geometric mean and 95% CI). Moreover, even the authors of the study acknowledged that higher variability on the estimate of residual PT in aP vaccines may not have clinical consequences as serious as those associated with wP, due to the important difference in residual activity between the two types of vaccine (see Sections 1.1 and 5.3).

5.2. Use of a single PT positive control

Substantial advancements in product characterization and process development, control and monitoring have led to the possibility of notable improvements in vaccine batch-to-batch consistency. This has allowed the popularization of a principle in use since at least the mid-1990s. Recently identified as the "consistency approach" [6], this testing philosophy is based on the concept that perfected analytical tools and the use of quality systems to guarantee consistency in both production and testing methods help a manufacturer offer assurances that any batch manufactured post-licensure possesses characteristics similar to those batches already shown to be safe and effective in the target population. In this approach, test designs, including those using animals, are critically examined in the light of their ability, as a set, to predict safety and efficacy in the target population. Therefore, the extensive adoption of the framework may lead to a considerable reduction, refinement, or even replacement of the use of animals for vaccine testing.

A reduction alternative based on the consistency approach is the substitution of a multidose test with a single-dose test once it has been established through repeated testing that good quality product is being consistently produced and that required animal testing reliably displays desirable characteristics (e.g., linearity of the response). This reduction has already been adopted for the potency testing of diphtheria and tetanus toxoids [31].

Studies were performed in our laboratory to generate data in support of a reduction in the number of mice used in the HSA test by using a single positive control group rather than the three or more groups of mice originally used to calculate the HSD_{50}. Acceptance of this modification required experiments designed to verify that a prespecified dose of PT, at or below 100 ng, would consistently sensitize more than 50% of the mice injected. For this purpose, groups of mice of the three strains found sensitizable were injected with 100 ng of pure PT in five to ten tests performed according to the routine method, which includes a three-dose PT titration curve. This dose was found adequate to consistently sensitize more than 50% of the mice injected (**Table 6**).

The number of mice employed for HSA testing of U.S.-licensed vaccines containing an aP component has been reduced by persuading one manufacturer to switch to the use of a single positive control group for assay validity assessment on the basis of the above information and its own studies.

Three manufacturers of aP vaccines are licensed in the United States. One manufacturer has used a single-control assay since licensing of their first combination. In a 10-year period, 200 bulk lots of this vaccine were released for distribution, involving the sensitization of 1265 mice in control groups of the HSA. Another manufacturer had used an HSD_{50} control since the licensing of their first combination containing an aP component. In a period of almost 9 years, around 97 bulk lots of this vaccine were released for distribution, requiring the sensitization of 3171 mice in control groups of the HSA. The latter manufacturer was persuaded to use a single positive control group for assay validity assessment on the basis of their testing history, our experimental results, and their own data analysis. Approval of the use of a single control group in HSAs was granted to this manufacturer in 2008. As part of the approval, the manufacturer committed to verify the sensitizability of the mouse strain at least three times per year using the original multidose test for the positive control. Since approval of the simplified procedure, 14 to 20 mice are sensitized in the positive control group per test (vs. a median of 50 per test before the modification).

5.3. Quantitative HSA with a nonlethal endpoint

As part of the development of copurified aP vaccines in Japan, the need for a method to replace the traditional HSA was identified, because the existing procedure involving a lethal challenge was found to be not sensitive

enough to detect an expected tenfold reduction in residual PT activity, relative to that contained in wP vaccines [32]. An HSA developed by Ishida et al. [33] used change in mouse rectal temperature as the response, instead of death, following histamine challenge. This test was included in the Japanese Minimum Requirements of Biological Products in 1981. In this test, groups of ten mice each were intraperitoneally injected with 0.5 mL of dilutions of a reference wP vaccine (to which an activity in HSU/mL had been assigned) and the aP vaccine being tested. The mice were intraperitoneally challenged 4 days afterward with 4 mg of histamine dihydrochloride in 0.5 mL of saline. Rectal temperature was recorded 30 minutes after challenge using a thermocouple attached to an electronic recording device, and activity was calculated using a parallel line assay with temperature as the dependent variable and log dose as the independent variable. Lack of reversion to toxicity was assessed as above using vaccine that had been incubated for one month at 37ºC. Copurified aP Japanese vaccines passed the test if their PT activity did not exceed 0.8 HSU/mL. Clinical studies in the mid-1980s revealed that some Japanese aP vaccines were prone to reversion to PT toxicity over time. To prevent this from happening, the limit of acceptance was revised to no more than 0.4 HSU/mL in 1991; it remains as such to date. Changes in manufacturing that were made to ensure the meeting of this specification had a noticeable impact on the residual PT activity of pertussis concentrates used for further manufacturing of aP vaccines licensed in the United States for toddler use also in 1991.

A study of the agreement of testing outcomes for non-Japanese aP vaccines, using the Japanese and other assay designs (U.S., EU WHO), was recently published [34]. The rationale for the investigation was the claimed superiority of the measurement of body temperature instead of lethality as an index of histamine sensitization for the detection of lower amounts of active PT in aP vaccines. However, results were rather uninformative, because many current products give a negative response in the lethal HSA models [35]. In addition, the clinical superiority of a quantitative method over a limit test is not immediately evident. Although, in principle, the results of such a test could be used to establish a proportional relationship with adverse events in the target population, meaningful studies to this effect are very difficult, if at all possible, to implement. Nevertheless, a feasible advantage of a quantitative estimation of residual aP is that such a result may allow more precise tracking and trending of data to maintain product consistency, because it provides a result in an interval scale.

A simplification of the HSA nonlethal refinement has recently been described. In it, an infrared thermometer is used to measure a drop in dermal temperature following the histamine challenge, instead of the change in rectal temperature [35,36].

5.4. Use of a nonlethal endpoint in a limit HSA

The possibility of using a nonlethal endpoint such as a drop in body temperature may constitute an important refinement, even though at this time some deaths still occur before mice can be humanely sacrificed. Moreover, the need for a reference group in the test to obtain a quantitative estimate of residual PT activity increases the number of animals required relative to the number used in a simplified limit test.

A potential way to circumvent the latter conundrum is to adapt the nonlethal endpoint to the limit test format. This has been reported by a group at the Danish Staten Seruminstitut (Jensen, personal communication). In the procedure that they have validated, the researchers inject each of four groups of C57BL mice intraperitoneally with either two single human doses of aP-containing test vaccine, the same dose of a control aP, or the control aP spiked with 50 ng of PT and PBS-gelatin buffer. Five days after sensitization, mice are challenged with 1.2 mg of histamine, and the drop in dermal temperature is recorded 30 minutes after challenge. The assay is valid if the drop in temperature (signal) following histamine challenge in mice injected with the aP control does not differ significantly from that of mice injected with buffer and if signal due to injection with spiked aP is significantly different from that due to nonspiked aP. The vaccine passes the test if the signal due to its injection is less than or equal to that due to the injection of the aP control vaccine. The method has been shown to have greater sensitivity than the lethal test, being able to detect as low as 6.25 ng of active PT in a human dose of Danish aP vaccine.

5.5. Potential replacement of the HSA to detect residual PT activity in aP vaccines

The majority of the toxic effects of PT have been attributed to the A-catalyzed ADP-ribosylation of the α-subunits of signal-transducing guanine-nucleotide-binding proteins. This led to the proposal of using an assay to measure this enzymatic reaction as an *in vitro* replacement for the HSA. An assay based on the chromatographic

separation and measurement of an ADP-ribosylated fluorescent substrate [37] was standardized [38,39]. However, PT has two functionally distinct domains, and the B-oligomer not only mediates the entry of A into the host cell, but possesses activities on its own [3]. By limiting a test for residual PT activity to measuring the ADP-ribosylating activity present in the aP vaccine, toxic activities due to the B oligomer may be overlooked. Nevertheless, the clinical risk of this limitation is low, because the B oligomer lectin-like activities require substantially higher protein concentrations for their expression. However, another important disadvantage of such an approach is that vaccines that cannot impair cell function might still show residual enzymatic activity. This is due to the fact that PT detoxification is a complex process that involves modification of multiple protein sites. For example, it has been proposed that formaldehyde, used for PT detoxification, although reactive with several amino acids, reacts preferentially with the ε amino group of lysine [40], and there are no lysine residues in the A protomer, making it potentially more difficult to detoxify than the B oligomer. Because of these concerns, an enzyme-linked immunosorbent assay (ELISA) system to monitor the modification of the B oligomer by detoxifying agents was developed as a potential complement of the enzymatic assay [41]. The assay is reported to be able to detect the loss, upon detoxification, of the ability of PT to bind to defined oligosaccharides or glycoproteins in the presence of other antigen components of aP in combination vaccines.

Recently, Yuen et al. proposed a combination of results of these two *in vitro* assays as a replacement for the HSA [39]. Such a suggestion is premature. The authors of the initiative made a strenuous effort to find a correlation between the nonlethal version of the HSA and a combination of both *in vitro* outcomes. Even then, a common correlation could not be found for all types of vaccines studied. Additionally, it is important to recall that correlation between two methods is not equivalent to agreement between pairs of results [42]. For the two versions of the HSA, a common acceptance limit for all vaccines has been established in at least Japan (nonlethal outcome) and the United States (lethal outcome). In this context, the authors of the study chose to limit the comparison of the *in vitro* combined index to the HSA outcome obtained using the Japanese design, which has never been directly compared to the lethal endpoint design or anchored to clinical behavior of aP vaccines. There is at this point no indication on how an acceptance limit could be set for residual PT activity using the dual *in vitro* assay proposed.

6. Conclusions

Mice have provided an invaluable service for the release of pertussis vaccines with improved public acceptability. The HSA in its two designs (using a lethal and a nonlethal endpoint to measure sensitization) has served well to ensure the release of aP vaccines with acceptable residual PT activity. Encouraging progress has occurred in the path to reducing and refining the use of mice for the toxicity testing of this type of vaccine. However, substantial advancement could occur if the test were critically examined in the context of the consistency approach and if harmonization were sought in good faith on a global scale. The HSA could become a limit test that uses a single PT positive control and a nonlethal endpoint to indicate sensitization to histamine. The test can be humanely stopped before hypovolemic-hypotensive shock progresses to death. If tight control of PT inactivation is ensured in-process by an adequate *in vitro* method, the quantitative *in vivo* measurement of residual PT activity may not be warranted in light of the impossibility of establishing with numerical certainty the amount of PT that is clinically hazardous. Despite their limitations, steps towards the replacement of an *in vivo* toxicity test for aP vaccines with an *in vitro* test for residual PT activity in final bulks of aP vaccines are heartening. The chosen method does not need to be unduly complex but need only provide assurance that residual PT activity in all types of aP vaccine is below a level consistent with lots shown to be safe in the clinic. More research is required to reach universal consensus on such a level.

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